Plasma Membrane Ca-ATPase of Radish Seedlings[1]

I. Biochemical Characterization Using ITP as a Substrate

Antonella Carnelli, Maria I. De Michelis, and Franca Rasi-Caldogno*

Centro di Studio del Consiglio Nazionale delle Ricerche sulla Biologia Cellulare e Molecolare delle Piante, Dipartimento di Biologia, Università di Milano, Via Celoria 26, 20133 Milano, Italy (A.C., F.R.-C.); and Istituto di Botanica, Università di Messina, C.P. 58, 98166 Messina-S. Agata, Italy (M.I.D.M.)

ABSTRACT

In this work, we exploited the capability of the plasma membrane Ca-ATPase to utilize ITP as a substrate to study its characteristics in plasma membrane vesicles purified from radish (*Raphanus sativus* L.) seedlings. The majority of the ITPase activity of plasma membrane was Ca^{2+}-dependent. The Ca^{2+}-dependent ITPase activity was Mg^{2+}-dependent and was stimulated by the calcium ionophore A23187. It was inhibited by erythrosin B (concentration giving 50% inhibition, 50 nanomolar) and by vanadate (concentration giving 50% inhibition, 3 micromolar) and displayed a broad pH optimum around pH 7.2 to 7.5. Both the hydrolytic and the transport activity of the plasma membrane Ca-ATPase were half-saturated by Ca^{2+} in the micromolar concentration range. No major effect of EGTA on the saturation kinetics of the enzyme was observed. The affinity of the plasma membrane Ca-ATPase for Ca^{2+} was about fourfold higher at pH 7.5 than at pH 6.9. The Ca^{2+}-dependent ITPase activity was stimulated about twofold by polyoxyethylene 20 cetyl ether, although it was inhibited by Triton X-100 and by lysolecithin.

An increasing amount of evidence indicates that calcium plays an important role in many cellular processes in plants, by acting as a second messenger of endogenous or exogenous stimuli (18). Cytoplasmic Ca^{2+} homeostasis is the result of the activity of passive and active transport systems localized in the PM[2] as well as in different endomembranes (11, 25).

In the past few years, the availability of transport-competent PM vesicles has made it possible to demonstrate that active transport of Ca^{2+} through the PM of plant cells is catalyzed by a Ca-ATPase (for a review, see refs. 1, 7, 11). The transport activity of this enzyme has been characterized in some detail in membrane vesicles from different plant materials and in proteoliposomes reconstituted with proteins solubilized from

the PM (1, 7, 11 and references therein). It is a Mg^{2+}-dependent, vanadate-inhibited ATPase, characterized by low apparent K_m for ATP, low specificity for nucleoside triphosphate (ITP and GTP sustain Ca^{2+} uptake at about 50–70% the rate sustained by ATP), and broad, slightly alkaline pH optimum. Most of the reported values of apparent K_m for Ca^{2+} of the PM Ca-ATPase are in the micromolar range (13, 14, 27) with the noticeable exception of the enzyme of radish seedlings, for which we determined an apparent K_m value as low as 70 nM (21). The most peculiar characteristic of the PM Ca-ATPase perhaps is its high sensitivity to inhibition by EB. In native membranes, its activity is fully inhibited by 0.5 to 1 μM EB, a concentration that only slightly inhibits the activity of other membrane-bound ATPases of plants, including the PM H^+-ATPase (4, 20).

Demonstrating the hydrolytic activity of the PM Ca-ATPase in native PM vesicles has proven difficult, due to the simultaneous operation of the much more active PM H^+-ATPase, which is inhibited by Ca^{2+}, already in the micromolar range (5). Taking advantage of the different pH optima of the PM H^+-ATPase (approximately 6.5) and of the PM Ca-ATPase (7.0–7.5), we have shown that a Ca^{2+}-dependent ATPase activity inhibited by submicromolar EB is localized at the PM of radish seeds (21). The biochemical characteristics of this activity so far investigated, as well as its functional mol wt determined by radiation-inactivation, are very similar to those determined for ATP-dependent Ca^{2+} uptake into PM vesicles, indicating that it represents the hydrolytic activity of the PM Ca-ATPase (21–23). However, the simultaneous operation of the Ca^{2+}-inhibited PM H^+-ATPase is an important source of error in these measurements because any treatment can be expected to influence both activities in the same or in the opposite sense. Because the PM H^+-ATPase is highly specific for ATP as a substrate (5), it should be possible to use ITP or GTP as a substrate to measure the hydrolytic activity of the PM Ca-ATPase in native PM vesicles, minimizing complications arising from the simultaneous operation of the H^+-ATPase. Analysis of GTP-dependent Ca^{2+} uptake into PM vesicles from red beet has shown that GTP is a useful means to probe the transport function of the PM Ca-ATPase (27), but no data are available on its suitability to measure the hydrolytic activity of this enzyme. Because the presence of regulatory GTP-binding proteins in membranes from plant cells has been demonstrated (10), we have chosen to use ITP

[2] Abbreviations: PM, plasma membrane; EB, erythrosin B; BTP, bis-tris propane (1,3-bis(tris(hydroxymethyl)methylamino)-propane); K_a, association constant of the Ca-EGTA complex; K'_a, apparent association constant of the Ca-EGTA complex at specified pH; Brij 58, polyoxyethylene 20 cetyl ether; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; IC_{50}, effective concentration giving 50% inhibition.

as an alternative substrate to monitor the hydrolytic and transport activities of the PM Ca-ATPase.

In this paper, we describe the ITPase activity of PM purified from radish seedlings by the aqueous two-phase partitioning technique (8), and we show that Ca²⁺-dependent ITPase activity is a reliable measure of the hydrolytic activity of the PM Ca-ATPase. Moreover, we reconsider the dependence on Ca²⁺ concentration of the PM Ca-ATPase of radish and we show that the very low apparent K_m value we had previously reported (21) was due to the use of a too-high value of the association constant of the Ca-EGTA complex to calculate free Ca²⁺ concentrations. When correct determinations of free Ca²⁺ concentrations are made, the apparent K_m for Ca²⁺ of the PM Ca-ATPase of radish is in the micromolar range both in the absence and in the presence of EGTA and is influenced by pH in the physiological range of cytoplasmic pH values.

MATERIALS AND METHODS

Preparation of PM Vesicles

Radish seeds (*Raphanus sativus* L. cv Tondo Rosso Quarantino, Ingegnoli, Milano, Italy) were germinated for 24 h and stored at −80°C as previously described (8). Methods for microsome isolation and PM purification by phase partitioning in a 6.5% (w/w) Dextran T500 (Pharmacia, Uppsala, Sweden)-polyethylene glycol 3350 (Sigma) phase system containing 5 mM KCl and 5 mM potassium phosphate buffer (pH 7.8) have been described (8). Aliquots of PM suspension (1–2 mg protein mL⁻¹ in 0.25 M sucrose, 0.2% BSA [w/v], 0.5 mM PMSF, 0.5 mM DTT, 1 mM BTP-Mes, pH 6.0) were immediately frozen and kept at −80°C until use. Membrane proteins were assayed as described (8).

Determination of the Apparent Association Constant of the Ca-EGTA Complex

Free Ca²⁺ concentrations in Ca²⁺-EGTA buffers containing various concentrations of EGTA and CaCl₂ (0.4–2.0 mM), 40 mM BTP-Hepes (pH 6.9 or 7.5), 100 mM KBr, 50 mM KNO₃, 0.1 mM ammonium molybdate, were measured with a Radiometer F2112Ca Selectrode calibrated against CaCl₂ (Radiometer S3606) diluted in the same salt medium. The apparent association constants (K'_a) of the Ca-EGTA complex at the relevant pH values were computed on the basis of the equation:

$$K'_a = \frac{[\text{Ca-EGTA}]}{[\text{Ca}][\text{EGTA}]}$$

where Ca is measured free Ca²⁺, Ca-EGTA concentration is determined as the difference between total and free Ca²⁺ concentration, and EGTA is free EGTA, determined as the difference between total EGTA and Ca-EGTA concentration.

The values of K'_a so determined were 5 × 10⁵ ± 1.5 × 10⁴ M⁻¹ at pH 6.9 and 7.8 × 10⁶ ± 2 × 10⁶ M⁻¹ at pH 7.5. At pH 7.5, the K'_a values obtained from measured free Ca²⁺ are variable, due to the relatively low sensitivity of the electrode and to the high affinity of EGTA for Ca²⁺: free Ca²⁺ concentrations could be reliably determined only in buffers containing about equimolar concentrations of CaCl₂ and EGTA, *i.e.* in the equivalence zone, in which very small errors in CaCl₂ or EGTA concentration have dramatic effects on free Ca²⁺ concentration. If the measured K'_a at pH 6.9 is used, together with the protonization values α_1 2.4 × 10⁹ M⁻¹ and α_2 5.9 × 10⁸ M⁻¹ given by ref. 24 to determine the K_a value according to ref. 17, a value of 1.1 × 10¹⁰ M⁻¹ is obtained. With a K_a of 1.1 × 10¹⁰ M⁻¹, the K'_a at pH 7.5 would be 7.6 × 10⁶ M⁻¹, a value well in the range of the experimentally determined ones.

Measurement of Ca²⁺-Dependent ITPase Activity

Unless otherwise specified, PM (100–150 μg membrane protein mL⁻¹) were incubated for 60 to 90 min in 40 mM BTP-Hepes (pH 6.9 or 7.5), 100 mM KBr, 50 mM KNO₃, 3 mM MgSO₄, 0.1 mM ammonium molybdate, 1 μg mL⁻¹ olygomycin, 1 mM ITP, 75 μg mL⁻¹ Brij 58. Calcium was supplied as CaCl₂ in the absence or in the presence of 1 mM (pH 6.9) or 2 mM (pH 7.5) EGTA. Free Ca²⁺ concentrations in the EGTA-buffered media were calculated using a K'_a value of 5 × 10⁵ M⁻¹ at pH 6.9 and 7.6 × 10⁶ M⁻¹ at pH 7.5. In all cases, Ca²⁺-dependent ITPase activity was evaluated as the difference between the activity measured in the presence of Ca²⁺ and that measured in the presence of EGTA alone.

Brij 58 was added from a 20 mg mL⁻¹ stock solution made in 1 mM BTP-Hepes, pH 7, 1 mM DTT, and 50% (v/v) ethanol. Liberated Pi was determined colorimetrically as described (9).

Measurement of Ca²⁺ Uptake

Ca²⁺ uptake was assayed by the membrane filtration technique essentially as previously described (20, 21). Unless otherwise specified, PM vesicles (about 30 μg membrane protein) were preincubated for 30 min at 25°C in 0.3 mL of 40 mM BTP-Hepes, pH 6.9, 100 mM KBr, 50 mM KNO₃, 3 mM MgSO₄, 0.1 mM ammonium molybdate, 5 mM (NH₄)₂SO₄, 5 μM FCCP, 1 μg mL⁻¹ oligomycin, 1 mM EGTA, and CaCl₂ labeled with 0.15 kBq/nmol of ⁴⁵Ca²⁺ (1.2 GBq/mg, New England Nuclear) to give the specified free Ca²⁺ concentrations. Other additions are specified in the legends. ATP- or ITP-dependent Ca²⁺ uptake was initiated by addition of the nucleoside triphosphates (1 mM final concentration) and the membranes were further incubated for 1 min (in the presence of ATP) or 2.5 min (in the presence of ITP). The reaction was terminated by dilution of the sample with 4 mL of 4 mM BTP-Hepes (pH 6.9), 5 mM MgSO₄, 2 mM EGTA, 100 mM KBr, and 50 mM KNO₃. The filter was washed three times with the above medium, allowed to dry, and dissolved in 5 mL of Filter-Count (Packard). Radioactivity was measured in a Prias Liquid Scintillation counter. Radioactivity associated with the filters of samples incubated in the absence of nucleoside triphosphates was subtracted from all the values to evaluate ATP- or ITP-dependent Ca²⁺ uptake.

RESULTS

Suitability of ITP as a Substrate

Plasma membrane vesicles purified from radish seedlings by the aqueous two-phase partitioning technique (8) are able

CARNELLI *ET AL.*

to take up Ca^{2+} upon addition of ATP or ITP (Fig. 1), indicating that at least part of the vesicles are in the inside-out configuration. In agreement with previous observations on PM from radish and from other plant materials (1, 7, 11), the initial rate of Ca^{2+} uptake sustained by ITP is about 50% of that sustained by ATP. The rate of Ca^{2+} uptake declines with time; this decline is earlier for ATP-dependent than for ITP-dependent Ca^{2+} uptake, thus confirming that intravesicular Ca^{2+} accumulation controls the activity of the PM Ca-ATPase (21).

The capability of the PM Ca-ATPase to utilize ITP allows one to measure its activity independently of the activity of the PM H^+-ATPase. This is especially relevant when the hydrolytic activity of the PM Ca-ATPase is to be measured, the activity of the H^+-ATPase, which is much more abundant, is inhibited by Ca^{2+} (5).

Table I shows that the great majority of the ITPase activity of the PM is Ca^{2+}-dependent. The Ca^{2+}-dependent ITPase activity is strictly Mg^{2+}-dependent and stimulated by the calcium ionophore A23187, as expected for the activity of an enzyme involved in Ca^{2+} transport and controlled by the intravesicular Ca^{2+} concentration (see Fig. 1 and ref. 21). In the presence but not in the absence of A23187, the Ca^{2+}-dependent ITPase activity is linear with time for at least 90 min (data not shown).

Figure 2A shows the effect of different detergents on the Ca^{2+}-dependent ITPase activity. Triton X-100 is strongly inhibitory already at 50 μg mL^{-1} and its inhibiting effect increases with the increase of its concentration, so that the activity is virtually abolished by 200 μg mL^{-1} of the detergent. A more complex pattern is observed in the presence of lysolecithin. Here, the marked inhibition induced by as little as 20 μg mL^{-1} lysolecithin is followed by a partial recovery in the presence of 50 μg mL^{-1} of the detergent, probably due to
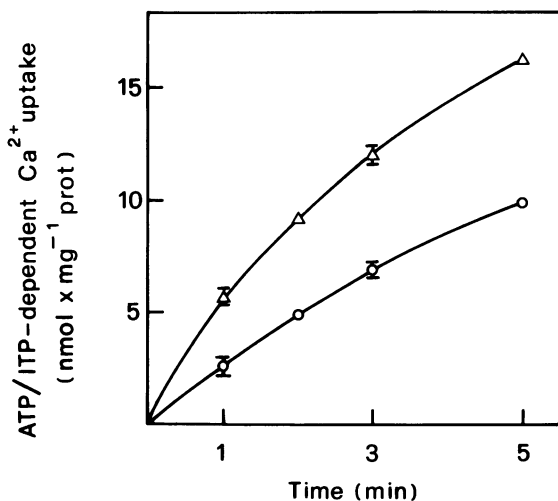
Table I. *Effect of Ca^{2+} and of the Calcium Ionophore A23187 on ITPase Activity*

Assays were performed at pH 6.9 as described in "Materials and Methods," but in the absence of Brij 58 and in the presence of 5 μM FCCP and 5 mM $(NH_4)_2SO_4$. Free Ca^{2+} was buffered at 100 μM with 1 mM EGTA; A23187 was supplied at 5 μM. Results are the mean of three experiments, each with three replicates, plus or minus the standard error of the mean.

Treatment	ITPase Activity		
	$-Ca^{2+}$	$+Ca^{2+}$	Ca^{2+}-dependent
	μmol mg^{-1} prot h^{-1}		
Control	0.23 ± 0.01	0.76 ± 0.02	0.53
+ A23187	0.22 ± 0.01	1.03 ± 0.03	0.81
- Mg^{2+}	0.07 ± 0.01	0.08 ± 0.01	0.01

unmasking of catalytic sites unaccessible to the substrate in right-side-out PM vesicles. The Ca^{2+}-dependent ITPase activity slowly declines with further increases of lysolecithin concentration. Among the detergents tested, only Brij 58 stimulates the Ca^{2+}-dependent ITPase activity: under our assay conditions, Brij 58 in the 20 to 200 μg mL^{-1} concentration range roughly doubles the Ca^{2+}-dependent ITPase activity. If the effect of Brij 58 is, as suggested, only the unmasking of Ca-ATPase catalytic sites, about 50% of vesicles are right-side-out. Figure 2B shows that the activating effect of 75 μg mL^{-1} of Brij 58 is independent of membrane concentration in the assay, so that the Ca^{2+}-dependent ITPase activity is proportional to membrane concentration in the assay up to 160 μg mL^{-1} also in the presence of the detergent.

Figure 3 shows that the Ca^{2+}-dependent ITPase activity is fully inhibited by EB (IC$_{50}$ 50 nM) and by vanadate (IC$_{50}$ 3 μM). The IC$_{50}$ of vanadate for inhibition of the Ca^{2+}-dependent ITPase activity is about one order of magnitude lower than that reported for the transport activity of the PM Ca-ATPase (13, 20, 27). This discrepancy most likely arises from the different experimental conditions used for measuring the hydrolytic or the transport activity. In fact, Ca^{2+} uptake was measured over 5 min of incubation (13, 20, 27), whereas the Ca^{2+}-dependent ITPase in Figure 3 was measured over 1 h of incubation, and it is known that the inhibiting effect of vanadate is strongly time-dependent (9). Moreover, in the experiment of Figure 3, vanadate access to the enzyme might have been facilitated by the presence of the detergent Brij 58.

The Ca^{2+}-dependent ITPase activity has a broad pH optimum around pH 7.2 to 7.5 (Fig. 4).

Dependence on Ca^{2+} Concentration

To ascertain whether the very low value of apparent K_m we had previously determined for the PM Ca-ATPase of radish seedlings was a characteristic of radish, or rather had to be ascribed to the use of EGTA-buffered media, in a first set of experiments the dependence of the PM Ca-ATPase activity on the concentration of Ca^{2+} was assayed at the same pH (7.5) of the previous experiments (21), but in the absence of EGTA. The results reported in Figure 5A (squares) show that at pH 7.5, the Ca^{2+}-dependent ITPase increases with the increase of $CaCl_2$ concentration in the medium, being satu-
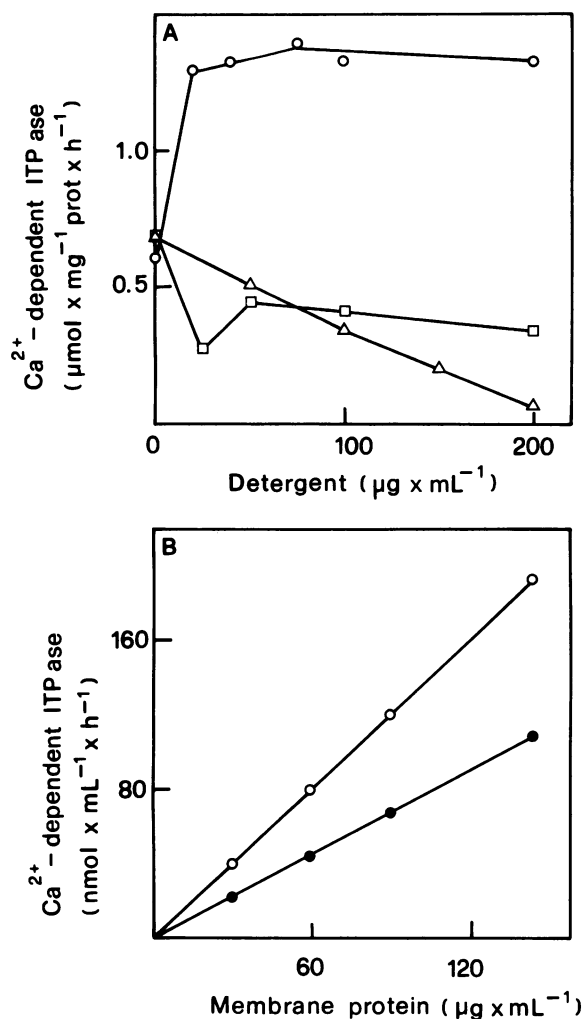

Figure 1. Time course of ATP-($\triangle$) and ITP-(O) dependent Ca^{2+} uptake into PM vesicles purified from radish seedlings. Ca^{2+} uptake was measured as described in "Materials and Methods": free Ca^{2+} was buffered at 10 μM with 1 mM EGTA. Results are from three experiments, each with three replicates; bars are the standard error of the mean.

Figure 2. Effect of detergents on the Ca^{2+}-dependent ITPase activity. Assays were performed at pH 6.9 as described in "Materials and Methods," but in the presence of 5 μM A23187, in the presence of the specified concentrations of Triton X-100 ($\triangle$), lysolecithin ($\square$), or Brij 58 ($\bigcirc$), with 100 μg membrane protein mL^{-1} (A). Free Ca^{2+} was buffered at 100 μM with 1 mM EGTA. In the experiments on Brij 58, all the samples contained 10 μM DTT and 0.5% (v/v) ethanol. Results are from three experiments, each with three replicates; bars are the standard error of the mean. In B, the effect of 75 μg mL^{-1} of Brij 58 was analyzed in the presence of the indicated membrane concentrations ($\bullet$, control; $\bigcirc$, plus Brij 58).

rated in the presence of 50 to 100 μM CaCl$_2$. The activity is half-maximal at about 2 μM CaCl$_2$. This value is about 30 times higher than that previously measured in an EGTA-buffered medium (70 nM in ref. 21). This discrepancy could be explained if the actual free Ca^{2+} concentrations in the EGTA-buffered media were much higher than those computed according to ref. 17 using a value of K_a of the Ca-EGTA complex of 10^{11} M^{-1} (24). The K_a values of the Ca-EGTA complex reported in the literature are quite variable and are affected by the ionic composition of the medium (15, 24, 28).

As detailed in "Materials and Methods," we have experimentally determined the value of the K_a of the Ca-EGTA

complex in our assay conditions, which is about 10-fold lower than reported by ref. 24 and used in our previous work (21), but similar to that reported by other authors (28). If the experimentally determined value of K_a is used to calculate free Ca^{2+} concentrations according to ref. 17 in media buffered with 2 mM EGTA at pH 7.5, the Ca^{2+}-dependent ITPase activity is half-saturated by about 3 μM free Ca^{2+} (data not shown), which is very similar to the concentration of CaCl$_2$ that semisaturates the activity in the absence of EGTA (Fig. 5A, squares). However, determination of the dependence of the activity on free Ca^{2+} concentration in EGTA-buffered media at pH 7.5 is subject to an important error because, in the presence of 2 mM EGTA, the relevant free Ca^{2+} concentrations (3–30 μM) are obtained by changing CaCl$_2$ concentrations as little as from 1.92 to 2.02 mM.

In the micromolar free Ca^{2+} concentration range, the Ca^{2+}-EGTA buffer system can be used much more reliably at pH 6.9, where the K'_a is about one-tenth that at pH 7.5 and CaCl$_2$ has to be varied from 0.6 to 0.97 mM to vary free Ca^{2+} between 3 and 30 μM in the presence of 1 mM EGTA. To further check whether the apparent affinity of the PM Ca-ATPase for Ca^{2+} was affected by EGTA, we have compared the dependence of Ca^{2+}-dependent ITPase activity on free Ca^{2+} concentration in EGTA-buffered medium (Fig. 5B) and on CaCl$_2$ concentration in the absence of EGTA (Fig. 5A, circles) at pH 6.9, which is still in the pH range of optimal activity of the PM Ca-ATPase (Fig. 4). Figure 5B shows also the dependence on free Ca^{2+} concentration in the EGTA-buffered medium of ITP-dependent Ca^{2+} uptake. The results show that EGTA has no major effect on the apparent affinity of the PM Ca-ATPase for Ca^{2+}: about 8 μM CaCl$_2$ is required to semisaturate the Ca^{2+}-dependent ITPase activity in the
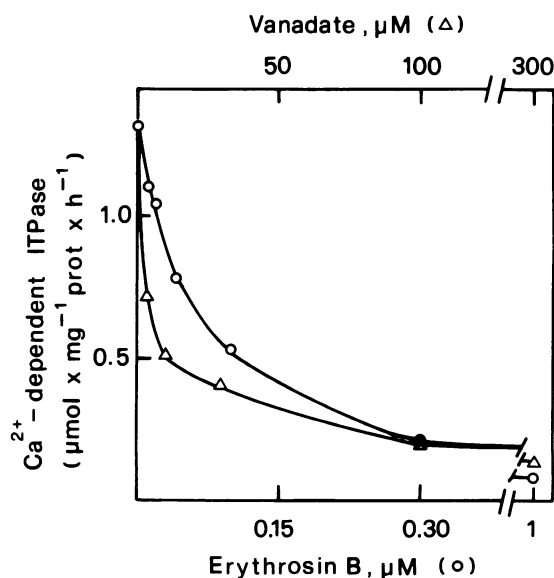

Figure 3. Effect of vanadate and of EB on the activity of the Ca^{2+}-dependent ITPase. Assays were performed at pH 6.9 as described in "Materials and Methods" in the presence of the indicated concentrations of vanadate and EB. Free Ca^{2+} was buffered at 100 μM with 1 mM EGTA.

absence of EGTA, and respectively 12 and 8 μM free Ca^{2+} to half-saturate Ca^{2+}-dependent ITPase activity or ITP-dependent Ca^{2+} uptake in the EGTA-buffered media. Similar results were obtained when Ca^{2+} uptake was energized with ATP (data not shown).

Data in Figure 5A show that the dependence of the PM Ca-ATPase activity on Ca^{2+} concentration is markedly influenced by the pH of the assay medium, the concentration of Ca^{2+} required to half-saturate the enzyme being much lower at pH 7.5 than at pH 6.9.

DISCUSSION AND CONCLUSIONS

The results reported in this paper confirm and extend the previously reported observation that the low specificity for nucleoside thiphosphates of the PM Ca-ATPase can be fruitfully exploited in the study of its biochemical and regulatory properties (27). In particular, we show that the Ca^{2+}-dependent ITPase activity of the PM is a reliable measurement of the hydrolytic activity of the PM Ca-ATPase. In fact, the bulk of the ITPase activity of the PM is Ca^{2+}-dependent and presents characteristics of Mg^{2+} and pH dependence, stimulation by the Ca^{2+} ionophore A23187, sensitivity to inhibition by vanadate and by EB, which clearly show that it represents the hydrolytic activity of the PM Ca-ATPase responsible for Ca^{2+} extrusion through the PM of plant cells.

The study of the activity of the PM Ca-ATPase using ITP (this paper) or GTP (27) as a substrate has been and will be very useful because it allows a study of its activity in native membranes under conditions in which the PM H^+-ATPase is not active, and the Ca-ATPase is the only operating ATPase.

In this paper, we have exploited this possibility to get some insight into the relation between the enzyme and other components of the membrane by analyzing the effect of detergents on the Ca^{2+}-dependent ITPase activity. In PM vesicles isolated by the aqueous two-phase partitioning technique, low concen-
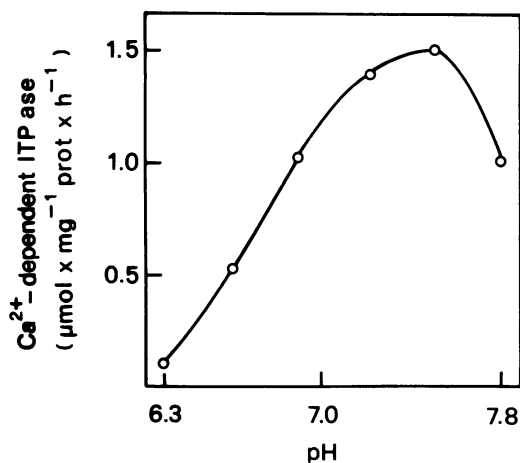

Figure 5. Dependence of Ca^{2+}-dependent ITPase activity (O, ●, ■) and of ITP-dependent Ca^{2+} uptake (△) at pH 6.9 (O, ●, △) and at pH 7.5 (■) on the concentration of Ca^{2+} in the absence (A) or in the presence (B) of 1 mM EGTA. Results are from three experiments, each with three replicates; bars are the standard error of the mean.

trations of detergents are expected to increase the activity of enzymes hydrolyzing nonpermeant substrates such as nucleoside triphosphates by making accessible to the substrate catalytic sites exposed to the intravesicular volume in sealed right-side-out PM vesicles (16). The unmasking effect of lysolecithin and Triton X-100 on the PM Ca-ATPase activity is largely, if not completely, overcome by the drastic inactivation of the enzyme. Among the detergents tested, only Brij 58 stimulates the Ca^{2+}-dependent ITPase activity in PM vesicles from radish. This behaviour of Brij 58 can probably be ascribed to its inability to remove essential lipids bound to the hydrophobic region of the Ca-ATPase (6, 16). This property, together with the fact that its effect on the PM Ca-ATPase is independent of its concentration as well as of the protein-to-detergent ratio in a fairly wide range, make Brij 58 a potential useful tool for the solubilization of the enzyme from PM in its active state (14, 16).

A second interesting point developed in this paper is the

Figure 4. Dependence of the Ca^{2+}-dependent ITPase activity on the pH of the assay medium. Ca^{2+}-dependent ITPase activity is the difference between the activity measured in the presence of 20 μM $CaCl_2$, without EGTA, and that measured in the presence of 1 mM EGTA. The medium was buffered at the indicated pH values with 40 mM BTP-Mes (pH 6.3 and 6.6) or BTP-Hepes (pH 6.9–7.8).
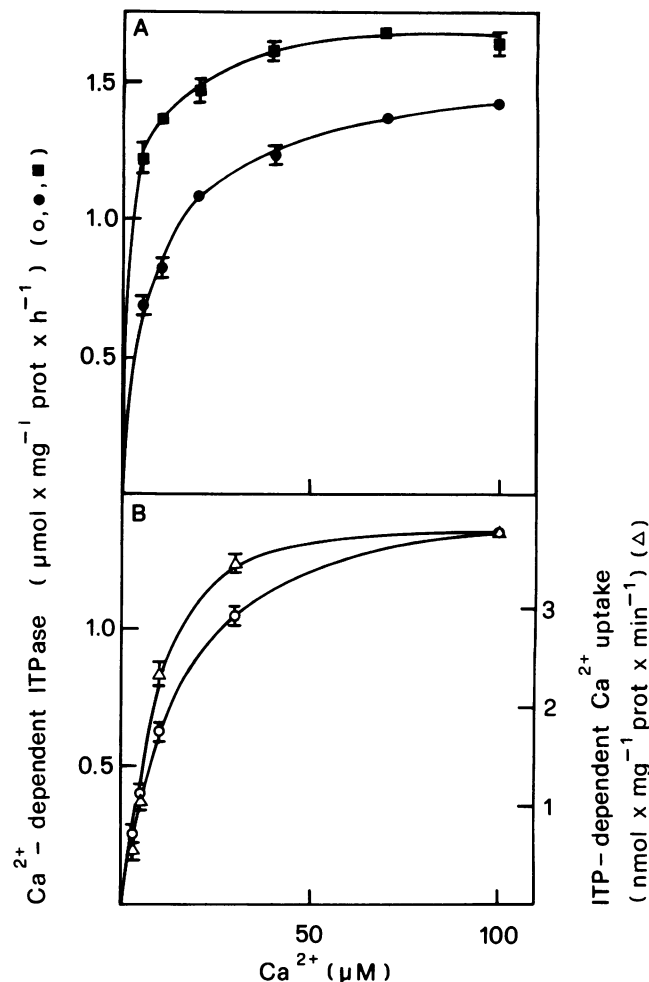
analysis of the dependence of the PM Ca-ATPase activity on the concentration of Ca^{2+}. The reported results show that, in agreement with that reported for the enzyme of other plant materials (13, 14, 27), the PM Ca-ATPase of radish presents saturation kinetics for Ca^{2+} and is half-saturated by Ca^{2+} concentrations in the micromolar range. The dependence of the enzyme activity on Ca^{2+} concentration is nearly the same when measured in the absence or in the presence of EGTA, provided that free Ca^{2+} concentrations in EGTA-buffered media are correctly evaluated. The apparent affinity for Ca^{2+} of the plant PM Ca-ATPase is markedly affected by pH in the physiological range of cytoplasmic pH values: the concentration of Ca^{2+} required to semisaturate the PM Ca-ATPase increases from 2 to 3 to 8 to 12 μM when the pH is lowered from 7.5 to 6.9. This is a further similarity of the plant enzyme with the erythrocyte Ca-ATPase (3, 7, 11), the affinity of which for Ca^{2+} is also pH-dependent in the same sense (26).

From a physiological point of view, the fact that saturation of the PM Ca-ATPase is reached well above the physiological cytoplasmic free Ca^{2+} concentration implies that the PM Ca-ATPase activity can increase dramatically in response to a stimulus-induced increase of cytoplasmic Ca^{2+}, and thus can play an important role in the reestablishment of the resting level of cytoplasmic Ca^{2+}. The pH sensitivity of the PM Ca-ATPase affinity for Ca^{2+}, and thus of its activity at physiological Ca^{2+} concentrations, might constitute a linkage between Ca^{2+} and pH homeostasis in the plant cell (2, 12). These points will be discussed further in the accompanying paper with respect to the role of calmodulin in regulating the activity of the PM Ca-ATPase (19).

LITERATURE CITED

1. Briskin DP (1990) Ca^{2+}-translocating ATPase of the plant plasma membrane. Plant Physiol 94: 397–400
2. Busa WB, Nuccitelli R (1984) Metabolic regulation via intracellular pH. Am J Physiol 246: R409–R438
3. Carafoli E (1991) Calcium pump of the plasma membrane. Physiol Rev 71: 129–153
4. Cocucci MC (1986) Inhibition of plasma membrane and tonoplast ATPases by erythrosin B. Plant Sci 47: 21–27
5. Cocucci MC, Marrè E (1984) Lysophosphatidylcholine-activated, vanadate-inhibited, Mg^{2+}-ATPase from radish microsomes. Biochim Biophys Acta 771: 42–52
6. De Foreste B, Le Maire M, Orlowski S, Champeil P, Lund S, Moller V, Michelangeli F, Lee AG (1989) Membrane solubilization by detergent: use of brominated phospholipids to evaluate the detergent-induced changes in Ca^{2+}-ATPase/lipid interaction. Biochemistry 28: 2558–2567
7. De Michelis MI, Rasi-Caldogno F, Pugliarello MC (1992) The plasma membrane Ca^{2+} pump: potential role in Ca^{2+} homeostasis. In CM Karssen, LC Van Loon, D Vreugdenhil, eds, Progress in Plant Growth Regulation. Kluwer Academic Publishers, Dordrecht, The Netherlands (in press)
8. De Michelis MI, Rasi-Caldogno F, Pugliarello MC, Olivari C (1991) Fusicoccin binding to its plasma membrane receptor and the activation of the plasma membrane H^+-ATPase. II.

Stimulation of the H^+-ATPase in a plasma membrane fraction purified by phase-partitioning. Bot Acta 104: 265–271
9. De Michelis MI, Spanswick RM (1986) H^+-pumping driven by the vanadate-sensitive ATPase in membrane vesicles from corn roots. Plant Physiol 81: 542–547
10. Drobak BK, Allan EF, Comerford JG, Roberts K, Dawson AP (1988) Presence of guanine nucleotide-binding proteins in a plant hypocotyl microsomal fraction. Biochem Biophys Res Commun 150: 899–903
11. Evans DE, Briars SA, Williams LE (1991) Active calcium transport by plant cell membranes. J Exp Bot 236: 285–303
12. Felle H (1988) Auxin causes oscillations of cytosolic free calcium and pH in Zea mays coleoptiles. Planta 174: 495–499
13. Giannini JL, Ruiz-Cristin J, Briskin DP (1987) Calcium transport in sealed vesicles from red beet (Beta vulgaris L.) storage tissue. II. Characterization of $^{45}Ca^{2+}$ uptake into plasma membrane vesicles. Plant Physiol 85: 1137–1142
14. Gräf P, Weiler EW (1990) Functional reconstitution of an ATP-driven Ca^{2+}-transport system from the plasma membrane of Commelina communis L. Plant Physiol 94: 634–640
15. Harafuji H, Ogawa Y (1980) Re-examination of the apparent binding constant of ethylene glycol bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid with calcium around neutral pH. J Biochem 87: 1305–1312
16. Palmgren MG, Sommarin M, Ulvskov P, Larsson C (1990) Effect of detergents on the H^+-ATPase activity of inside-out and right-side-out plant plasma membrane vesicles. Biochim Biophys Acta 1021: 133–140
17. Pershadsingh HA, McDonald JM (1980) A high affinity calcium-stimulated magnesium-dependent adenosine triphosphatase in rat adipocyte plasma membrane. J Biol Chem 255: 4087–4093
18. Poovaiah BW, Reddy ASN (1987) Calcium messenger system in plants. CRC Crit Rev Plant Sci 6: 47–103
19. Rasi-Caldogno F, Carnelli A, De Michelis MI (1992) Plasma membrane Ca-ATPase of radish seedlings. II. Regulation by calmodulin. Plant Physiol 98: 1202–1206
20. Rasi-Caldogno F, Pugliarello MC, De Michelis MI (1987) The Ca^{2+}-transport ATPase of plant plasma membrane catalyzes a nH^+/Ca^{2+} exchange. Plant Physiol 83: 994–1000
21. Rasi-Caldogno F, Pugliarello MC, Olivari C, De Michelis MI (1989) Identification and characterization of the Ca^{2+}-ATPase which drives active transport of Ca^{2+} at the plasma membrane of radish seedlings. Plant Physiol 90: 1429–1434
22. Rasi-Caldogno F, Pugliarello MC, Olivari C, De Michelis MI (1989) The calcium pump of the plasma membrane of higher plants. In J Dainty, MI De Michelis, E Marrè, F Rasi-Caldogno, eds, Plant Membrane Transport: The Current Position. Elsevier, Amsterdam, pp 225–230
23. Rasi-Caldogno F, Pugliarello MC, Olivari C, De Michelis MI, Gambarini G, Colombo P, Tosi G (1990) The plasma membrane Ca^{2+}-pump of plant cells: a radiation inactivation study. Bot Acta 103: 39–41
24. Sillen LG, Martell AE (1971) Stability Constants of Metal-Ion Complexes. Special publications Nos. 17 and 25, The Chemical Society, Burlington House, London
25. Tester M (1990) Plant ion channels: whole-cell and single-channel studies. New Phytol 114: 305–340
26. Villalobo A, Brown L, Roufogalis BD (1986) Kinetic properties of the purified Ca^{2+}-translocating ATPase from human erythrocyte plasma membrane. Biochim Biophys Acta 854: 9–20
27. Williams LE, Schueler SB, Briskin DP (1990) Further characterization of the red beet plasma membrane Ca^{2+}-ATPase using GTP as an alternative substrate. Plant Physiol 92: 747–754
28. Wolf HU (1973) Divalent metal ion buffers with low pH-sensitivity. Experientia 29: 241–249